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Expression in *Escherichia coli* and purification of biologically active L proteinase of foot-and-mouth disease virus

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Abstract

The foot-and-mouth disease virus (FMDV) Lb gene was cloned into bacterial expression vectors under the control of a T7 RNA polymerase promoter. The Lb protein was expressed in both an in vitro transcription-translation system and in *Escherichia coli*. In vitro expression of a construct containing the *Lb* gene fused to a portion of the *VP4* and *3D* genes demonstrated *cis* cleavage activity that could be blocked by the thiol protease inhibitor E-64. Lb expressed in *E. coli* was purified from the soluble fraction by metal chelation chromatography. Purified Lb had *trans* cleavage activity at the L/P1 junction and cleaved the p220 component of the cap-binding protein complex.

Keywords: Foot-and-mouth disease virus; Leader proteinase; Autocatalytic cleavage; p220 cleavage

1. Introduction

Foot-and-mouth disease virus (FMDV) is a member of the Picornaviridae and contains a single-stranded RNA genome. Subsequent to cell attachment and virus uncoating, the virion RNA is translated into a polyprotein which is processed during its synthesis, via a series of *cis* and *trans* cleavages, into individual gene products by three virus encoded proteinases, leader (L), 2A, and 3C.

The initial processing event within the polyprotein occurs when the L proteinase autocatalytically cleaves the L/P1 junction (Strebel and Beck, 1986). The L protein has also been implicated in the cleavage of p220, a subunit of the cap-binding

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protein complex, eIF-4, involved in the initiation of translation at the 5′ end of capped mRNAs (Devaney et al., 1988). Since picornavirus mRNAs are not capped, cleavage of this factor does not effect their translation but does result in the shutoff of most host cell protein synthesis (Etchison et al., 1982; Lloyd et al., 1988). In some other picornaviruses, i.e., poliovirus, it has been demonstrated that proteinase 2A is indirectly involved in the cleavage of p220 (Lloyd et al., 1986; Krausslich et al., 1987), although very recently it has been shown that both Coxsackie virus and rhinovirus 2A can directly cleave p220 (Lamphear et al., 1993). It is unclear whether FMDV L plays a direct or indirect role in the cleavage of p220.

Within the picornaviruses, only the aphthovirus (FMDV) and cardiovirus genera encode L proteins. Unlike that of FMDV, the cardiovirus L protein is processed from the P1 precursor by the 3C proteinase (Parks et al., 1986). In addition, in cardiovirus infected cells, p220 is not cleaved (Mosenkis et al., 1985; Lloyd et al., 1988). No definitive function has yet been assigned to the cardiovirus L protein, although recently Kong et al. (1994) suggested that the L protein of Theiler's virus may be involved in host cell-restricted virus expression.

Translation of FMDV RNA initiates at either of two in-frame AUG codons resulting in the synthesis of two L proteins, Lab and Lb, differing only at their amino terminus by 28 amino acids (Sangar et al., 1987). Lb, the smaller of the two proteins, is the major species synthesized in infected cells and in a cell-free system. Both proteins exhibit autocatalytic as well as p220 cleavage activity (Strebel and Beck, 1986; Devaney et al., 1988; Medina et al., 1993). The L protein has been implicated, by amino acid sequence alignment, to be a thiol protease related to the papain family of proteases (Gorbalenya et al., 1991). We have shown that E-64, a specific inhibitor of thiol proteases including the papain family of cysteine proteases (Hanada et al., 1978), blocks the autocatalytic cleavage of L from the P1-2A precursor in a cell-free system and an uncharged analog, E-64d, blocks autocatalytic and p220 cleavage in infected cells (Kleina and Grubman, 1992). Further, we demonstrated that E-64d blocks virus assembly and as a consequence reduces virus yield suggesting that this or a related compound might be an effective agent against FMD.

To examine the biochemical characteristics of the L protein and to identify compounds that specifically inhibit L functions, we were interested in expressing and purifying the L protein. In this communication, we describe the expression of the Lb gene and demonstrate *cis* and *trans* cleavage at the L/P1 junction and p220 cleavage activity of crude bacterial extracts as well as purified Lb protein.

2. Materials and methods

2.1. Plasmid construction

Plasmid pLb-VP4′-3D′ was derived from pTLP123 (Vakharia et al., 1987) by digestion with NcoI and religation (Fig. 1). This construct contains the complete

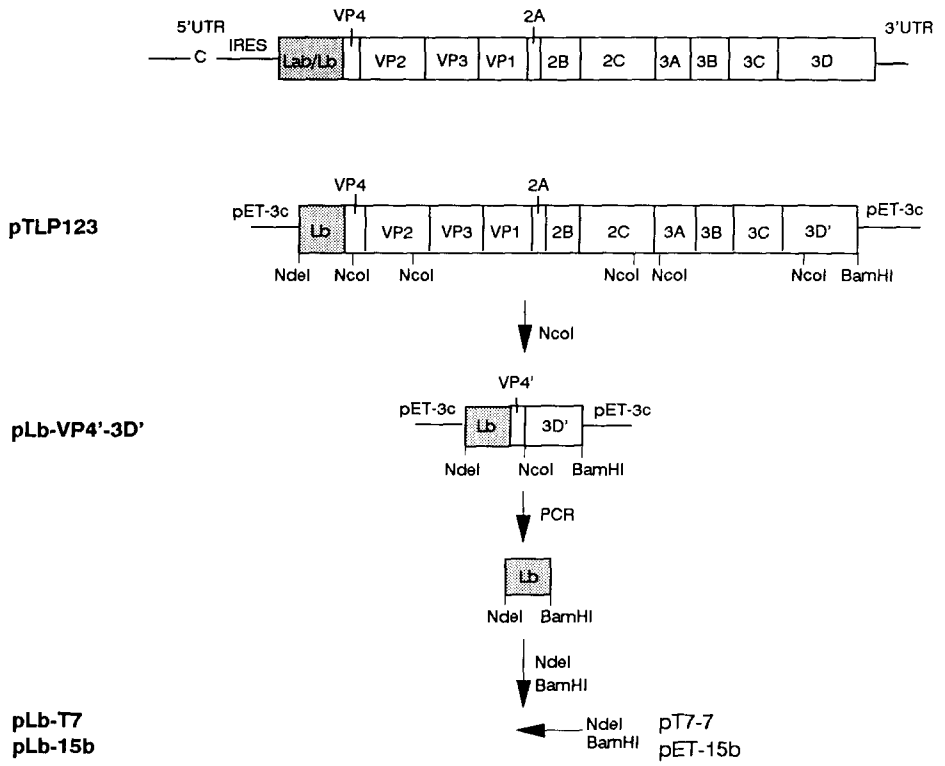

Fig. 1. Schematic representation of FMDV genome and construction of expression plasmids. The FMDV genome including the 5′ untranslated region (UTR), containing a poly(C) tract (C), and the internal ribosome entry site (IRES), the protein coding region, and the 3′ UTR are indicated. All constructs are under the control of a T7 RNA polymerase promoter. Plasmid pLb-VP4′-3D′ was constructed from pTLP123 by *Nco*I digestion and religation. The *Lb* gene was constructed by PCR and contains ATG start and TAG stop codons. The prime (′) denotes a truncated protein.

Lb gene, 33 amino acids of *VP4* and 163 amino acids of *3D*. An expression plasmid containing the *Lb* gene was constructed by the polymerase chain reaction (PCR) using linearized pLb-VP4′-3D′ (Fig. 1) as template, a primer representing the 5′ end of the *Lb* gene containing an *Nde*I restriction site (underlined) with an in-frame start codon (ATG), 5′-AAGGTCGTCATATGGAATTCACGCTG-TACAACGGC-3′, and a primer representing the 3′ end of the gene with a termination codon (TAG) followed by a *Bam*HI restriction site (underlined), 5′-ACCCGGATCCCTACTTAAGCTTTCGCTGAACGTT-3′. The purified PCR product was digested with *Nde*I and *Bam*HI, ligated into either *Nde*I-*Bam*HI digested pT7-7 or pET-15b (Novagen) and transformed into *E. coli* HB101 cells (Fig. 1). Plasmids containing the *Lb* gene, pLb-T7 and pLb-15b, respectively, were completely sequenced using Sequenase Version 2.0 (US Biochemicals). There were differences in the *Lb* gene compared to the published sequence at residues 94 (alanine to serine), 95 (arginine to glutamic acid), 96 (an additional amino acid,

valine), and 103 (asparagine to aspartic acid) (Robertson et al., 1985). Identical results were obtained when the *Lb* gene in the full-length infectious cDNA clone, $pRMC_{35}$ (Rieder et al., 1993), was sequenced. The additional amino acid resulted in an Lb protein of 173 amino acids. Plasmid $pRMC_{35}\Delta L$ (kindly provided by P. Mason, Plum Island Animal Disease Center) was derived from $pRMC_{35}$ and contains a deletion of 189 nucleotides in the *L* gene resulting in a truncated L protein that lacks amino acids 35–98.

2.2. Expression and purification of Lb in E. coli

Plasmid pLb-T7 was transformed into BL21(DE3)pLysE cells and pLb-15b was transformed into BL21(DE3) cells. These cells contain the T7 RNA polymerase gene in their chromosome that is inducible with isopropyl-β-D-thiogalactopyranoside (IPTG) (Studier and Moffat, 1986; Studier, 1991). BL21(DE3) cells carrying pLysE encode T7 lysozyme, which is an inhibitor of T7 RNA polymerase and reduces the ability of this polymerase to transcribe target genes in uninduced cells. Vector pET-15b codes for 19 amino acids including a stretch of 6 histidine residues that are expressed at the amino terminus of a target protein and allows proteins to be purified by metal chelation chromatography (Novagen). In addition pET-15b contains a *lac* operator sequence and the promoter and coding sequence for the *lac* repressor. These features significantly reduce the basal level of T7 RNA polymerase expression allowing the synthesis of many toxic gene products in BL21(DE3) cells.

Cultures of BL21(DE3)pLysE cells transformed with pLb-T7 were grown in the presence of ampicillin and chloramphenicol, induced with IPTG, and supernatant and pellet fractions were prepared and stored at −70°C and subsequently used in *trans* cleavage assays. The Lb protein was purified from BL21(DE3) cells transformed with pLb-15b. Cells were induced with IPTG for 2 h, centrifuged, resuspended in 5 mM imidazole, 500 mM NaCl and 20 mM Tris-HCl, pH 7.9 containing 0.1% Triton X-100 and 100 μg/ml lysozyme, incubated at 30°C for 15 min, and sonicated. The extract was centrifuged at 18000 rpm for 15 min. The Lb protein was purified from the soluble (supernatant) fraction by metal chelation chromatography using a column containing divalent cations (Ni^{2+}) immobilized on the His-Bind metal chelation resin and eluted with 1 M imidazole following the protocol recommended by Novagen. The purity of the Lb-15b protein was approximately 80% as determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Individual purified fractions were dialyzed into phosphate buffered saline (PBS), the protein concentration determined by the BCA protein assay (Pierce Chemical Co.), and the fractions stored in aliquots at −70°C in PBS containing 1 mM DTT and 50% glycerol.

2.3. Production of antisera

Purified Lb-15b was mixed with an equal volume of Freund's incomplete adjuvant and inoculated into rabbits or mixed with an equal volume of aluminum

hydroxide and inoculated into guinea pigs. Two booster inoculations were given. Serum was tested in a liquid phase radioimmunoprecipitation assay with radiolabeled cytoplasmic extracts from bovine kidney (LF-BK) cells infected with FMDV type A12.

2.4. *In vitro transcription and translation*

Transcripts from *Not*I linearized pRMC$_{35}\Delta$L were synthesized using T7 RNA polymerase and the MEGAscript kit (Ambion). Transcripts from *Nco*I or *Bam*HI linearized pLb-VP4′-3D′ or *Bam*HI linearized pLb-T7 were synthesized with T7 RNA polymerase and the mMESSAGE, mMACHINE capping kit (Ambion). Approximately 0.5–1 μg of RNA from transcription reactions or 1 μg FMD A12 virion RNA was used in a rabbit reticulocyte lysate in vitro translation system as

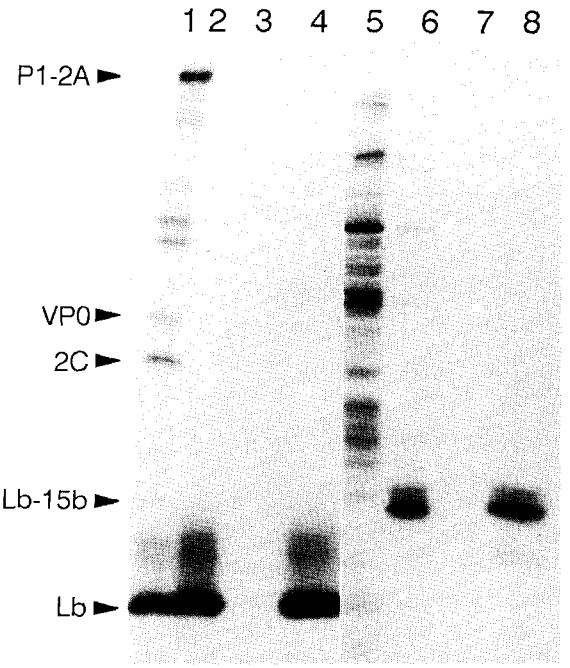

Fig. 2. In vitro and in vivo expression of Lb. RNA transcripts from *Bam*HI linearized pLb-T7 were translated in a cell-free system (lane 2), samples were immunoprecipitated with normal or L antiserum (lanes 3 and 4, respectively) and analyzed by SDS-PAGE on a 15% gel. Lane 1 is a cell-free translation programmed with FMD virion RNA. BL21(DE3) cells transformed with pLb-15b were induced as described in Materials and methods and radiolabeled with [^{35}S]methionine. Soluble fractions from uninduced (lane 5) or induced cells (lane 6) were analyzed by SDS-PAGE. Soluble fractions from induced cells were immunoprecipitated with normal or L antiserum (lanes 7 and 8, respectively).

previously described (Vakharia et al., 1987; Bablanian and Grubman, 1993). E-64 was purchased from Boehringer-Mannheim.

2.5. Immunoprecipitation

Radiolabeled in vitro translation products or *E. coli* expressed proteins were immunoprecipitated with anti-L serum (kindly provided by E. Beck, University of Heidelberg, Germany or produced in this study) and Protein G-bearing group G *Streptococcus* as previously described (Kleina and Grubman, 1992).

2.6. Trans cleavage assay

[^{35}S]Methionine-labeled translation products from transcripts of pRMC$_{35}\Delta$L were mixed with an equal volume of an extract from *E. coli* transformed with pLb-T7 or with different amounts of purified Lb-15b and incubated at 30°C, for varying periods of time. Products were analyzed by SDS-PAGE.

2.7. p220 cleavage assay

Postmitochondrial HeLa cell cytoplasmic extracts (S10) (Devaney et al., 1988) were incubated with purified Lb-15b and appropriate controls at 30°C overnight. Samples were analyzed by SDS-PAGE on a 7.5% minigel and transferred to a nitrocellulose membrane in a mini Trans-Blot electrophoretic cell (Biorad) for 1 h at 100 V. Blots were blocked, incubated overnight at room temperature with a 1 : 100 dilution of rabbit polyclonal antibody against p220 (kindly supplied by R. Lloyd, University of Oklahoma Health Science Center, Oklahoma City, OK), and detected with ^{125}I-labeled protein A as previously described (Devaney et al., 1988).

3. Results

3.1. In vitro and in vivo expression of Lb protein

The *Lb* gene produced by PCR was ligated into the expression vectors pT7-7 (pLb-T7) and pET-15b (pLb-15b). Plasmid pLb-T7 was linearized with *Bam*HI, transcribed and translated in a cell-free system (Fig. 2). The protein synthesized comigrated with Lb synthesized in an in vitro translation programmed with FMD virion RNA (Fig. 2, lanes 1 and 2) and was immunoprecipitated with L antiserum (lane 4). However, with the pT7-7 expression vector, synthesis of Lb protein occurred only in BL21(DE3)LysE cells and at a low level (data not shown). When the *Lb* gene was present in the vector pET-15b, which more efficiently controls the basal level of T7 RNA polymerase, the Lb protein was expressed in transformed BL21(DE3) cells. The Lb-15b protein was of higher molecular weight than Lb as a result of the fusion of 19 amino acids encoded by the vector to its amino terminus (lane 6) and was reactive with L antibodies (lane 8).

3.2. Autocatalytic cleavage by Lb

To examine Lb processing in *cis*, the plasmid pLb-VP4′-3D′ was linearized with either *Nco*I or *Bam*HI and RNA transcripts were translated. Transcripts synthesized from *Nco*I linearized plasmid code for the Lb protein and 33 amino acids of VP4, while transcripts from *Bam*HI linearized plasmid code for an additional 163 amino acids of 3D. In both cases translation resulted in the synthesis of Lb demonstrating that *cis* cleavage at the L/VP4 junction had occurred (Fig. 3, lanes 2 and 4). Translation of either transcript in the presence of E-64, which we have previously shown to block L/P1 cleavage in an in vitro translation assay programmed with virion RNA (Kleina and Grubman, 1992), also blocked *cis* cleavage in this system resulting in the synthesis of the expected larger products, i.e., Lb-VP4′ and Lb-VP4′-3D′, respectively (lanes 3 and 5).

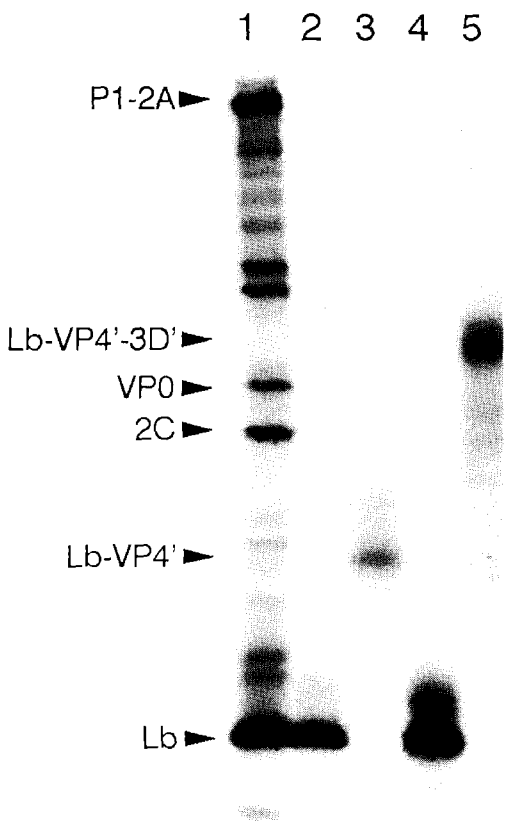

Fig. 3. *Cis* cleavage activity. RNA transcripts from *Nco*I or *Bam*HI linearized plasmid pLb-VP4′-3D′ were translated in a cell-free system in the absence (lanes 2 and 4) or presence of 480 μg/ml E-64 (lanes 3 and 5). The translation products were analyzed by SDS-PAGE on a 15% gel. Lane 1 is a cell-free translation programmed with FMD virion RNA.

3.3. Purification of Lb

A culture of 1 l of BL21(DE3) cells transformed with pLb-15b was grown, induced for 2 h and lysed. As shown in Fig. 4, the majority of the Lb-15b protein was present in the soluble fraction (lanes 3 and 4). The Lb-15b protein was purified from the soluble fraction by metal chelation chromatography. The major band, at approximately 25000 Da, eluted between fractions 6 and 8 (lanes 5–7) and comigrated with radiolabeled Lb-15b which was immunoprecipitated by L antiserum (data not shown). These fractions were combined, dialyzed against PBS and used to raise antisera. Approximately 0.5–1 mg purified Lb-15b was obtained from 1 l of cell culture. The eluted Lb-15b at fraction 7 (lane 6) was used in subsequent proteolytic assays.

3.4. Processing of the ΔL-P1 precursor by purified Lb

To determine if Lb proteinase produced in *E. coli* was able to cleave at the L/P1 junction in *trans*, a crude extract of Lb and column purified Lb-15b were

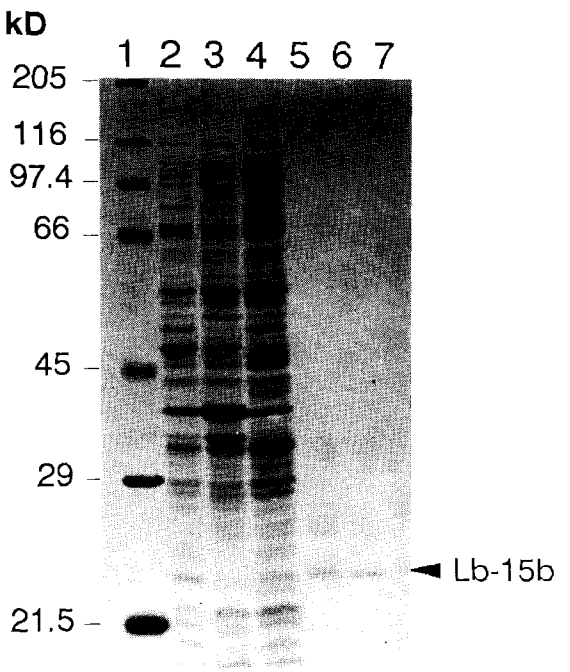

Fig. 4. Purification of FMDV Lb expressed in *E. coli*. BL21(DE3) cells transformed with pLb-15b were induced for 2 h, lysed, and Lb-15b purified from the soluble fraction by metal chelation chromatography as described in Materials and methods. Aliquots from the total cell lysate (lane 2), the insoluble fraction (lane 3), the soluble fraction (lane 4), and fractions 6–8 of the Lb purification scheme (lanes 5–7) were separated by SDS-PAGE on a 15% gel and visualized by staining with Coomassie brilliant blue. Molecular weight markers are shown on the left (lane 1).

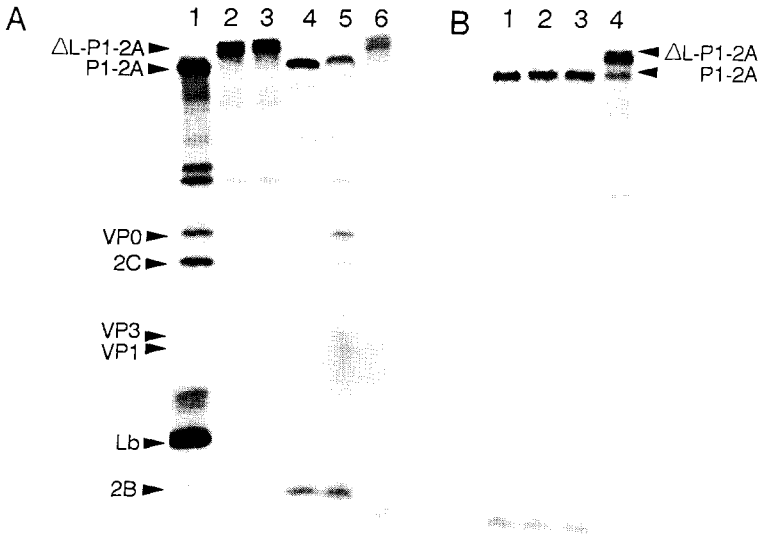

Fig. 5. Cleavage of the L/P1 junction by Lb. (A) [^{35}S]Methionine-labeled translation products of pRMC$_{35}\Delta$L transcripts (lane 2) were incubated for 1.5 h at 30°C with extracts of *E. coli* transformed with pT7-7 (lane 3), pLb-T7 (lane 4), and with S10 extracts from FMDV infected or mock-infected LF-BK cells (lanes 5 and 6, respectively) and examined by SDS-PAGE on a 15% gel. Lane 1 is a cell-free translation programmed with FMD virion RNA. (B) The same assay was performed with different amounts of purified Lb-15b (fraction 7) (412 ng, lane 1; 41.2 ng, lane 2; 4.12 ng, lane 3; and 0.41 ng, lane 4).

incubated with radiolabeled in vitro translation products of pRMC$_{35}\Delta$L transcripts. This plasmid contains a deletion in the *L* gene which results in a truncated L protein (ΔL) that cannot cleave itself at the L/P1 junction (Fig. 5A, lane 2). Addition of crude Lb to the radiolabeled translation products resulted in the *trans* cleavage of ΔL-P1-2A to P1-2A and ΔL (lane 4). The increase of a band in the 2B region might be the result of the apparent comigration of ΔL, which has a slightly smaller molecular weight. *Trans* processing is greater than 90% complete after a 15 min incubation (data not shown). When the translation mixture was incubated with a crude extract from pT7-7 induced cells or with an S10 fraction from mock-infected LF-BK cells no processing occurred (lanes 3 and 6). The released P1-2A precursor was partially processed to VP0, VP3, and VP1 by 3C synthesized in the in vitro translation (lane 4). Similar results, but more efficient processing of P1, was obtained with an S10 extract from FMDV infected LF-BK cells (lane 5) presumably because more 3C is present in the infected cell extract. Addition of E-64 (50 μg/ml) completely inhibited *trans* cleavage (data not shown).

From 0.4 to 400 ng of purified Lb-15b (fraction 7) was incubated with radiolabeled translation products from pRMC$_{35}\Delta$L transcripts (Fig. 5B). Addition of 4 ng of Lb-15b resulted in complete processing of ΔL-P1-2A, while 0.4 ng processed approximately 20% of the substrate (Fig. 5B, lanes 1–4).

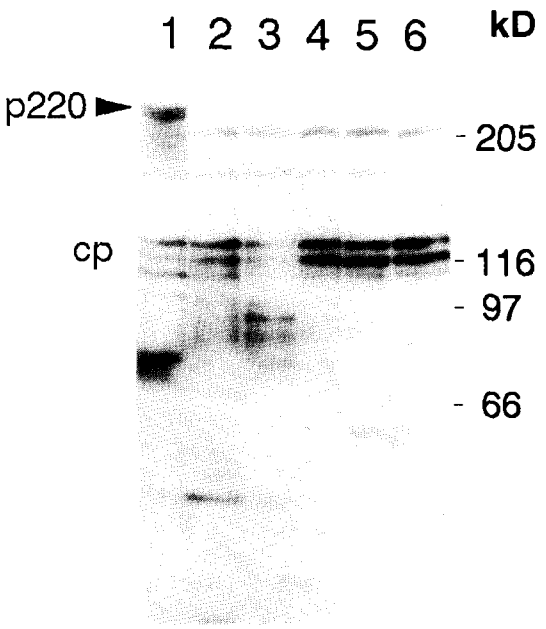

Fig. 6. Cleavage of p220 by Lb. HeLa S10 was incubated overnight at 30°C with extracts of *E. coli* transformed with pT7-7 (lane 1), pLb-T7 (lane 2) and with different amounts of purified Lb-15b (fraction 7) (2 μg, lane 3; 412 ng, lane 4; 41.2 ng, lane 5; and 4.12 ng, lane 6). The samples were electrophoresed on a 7.5% minigel and Western blot analysis performed as described in the text. Molecular weight markers are shown on the right. cp refers to cleavage products of p220.

3.5. p220 cleavage assay

We previously demonstrated that an FMDV infected S10 extract completely degraded p220 (Devaney et al., 1988; Lloyd et al., 1988). To assay for p220 cleavage activity a crude extract of Lb and from 4 to 2000 ng of purified Lb-15b (fraction 7) were incubated with HeLa S10 as a source of p220. Western blot analysis with polyclonal serum against p220 demonstrated that both crude Lb and 4 ng purified Lb-15b completely degraded p220 (Fig. 6, lanes 2–6), while 0.4 ng Lb-15b degraded approximately 90% of p220 (data not shown). A lysate from cells transformed with pT7-7 had no effect on p220 (lane 1).

4. Discussion

We have constructed, by PCR, the *Lb* gene of FMDV and expressed the gene product in *E. coli*. Since Lb is expressed at very low levels in the pT7-7 system, presumably because it is toxic to *E. coli*, we also used the pET-15b system which very tightly controls the basal level of expression of T7 RNA polymerase allowing

for more efficient expression of toxic proteins. In this latter system, Lb is expressed as a fusion protein containing additional amino acids at its amino terminus including a stretch of 6 histidine residues. This feature allows for one-step purification of Lb by metal chelation chromatography. Although purified Lb-15b contains additional residues, it as well as crude Lb has *trans* cleavage activity on a ΔL-P1-2A substrate that is unable to process itself at the L/P1 junction and also degrades the p220 component of the cap-protein binding complex. In addition, in vitro expression of the *Lb* gene fused to a portion of the *VP4* and *3D* genes demonstrated *cis* cleavage activity. The purified product is active at approximately 0.01–0.1 μM concentrations. The Lb-15b protein is predominantly present in the soluble fraction after treatment of lysed cells with Triton X-100 and sonication. We have obtained approximately 0.5–1 mg of purified Lb-15b from 1 l of induced cells. We also found that Lb-15b purified from the insoluble fraction by treatment with 6 M urea and metal chelation chromatography is as active as soluble Lb-15b (data not shown).

Utilizing a similar expression system Martinez-Abarca et al. (1993) have expressed and purified the 2A protein of poliovirus. This protein autocatalytically cleaves itself at the VP1/2A junction and is also involved in the cleavage of p220. In the system used, poliovirus 2A is predominantly found in the insoluble fraction of cells and in contrast to the relatively high specific activity of FMDV Lb, poliovirus 2A is much less active in both *trans* and p220 cleavage assays (Martinez-Abarca et al., 1993). Liebig et al. (1993) have purified, to near homogeneity, the 2A proteinase of both human rhinovirus 2 (HRV2) and Coxsackie virus B4 (CVB4). These proteins were expressed in a soluble form in *E. coli* and were shown to efficiently cleave peptides representing the VP1/2A cleavage site of the respective enzymes. Most interestingly both enzymes were shown to directly cleave the p220 component (eIF-4γ) of purified eIF-4. Additional work from this laboratory (Sommergruber et al., 1994) demonstrated that a peptide containing a consensus sequence of the HRV2 and CVB4 VP1/2A cleavage site is cleaved by both proteins. This consensus sequence is present in p220 and maps to the site of cleavage of p220 by HRV2 and CVB4 2A (Lamphear et al., 1993). Although no information is currently available concerning the essential residues in the L/P1 cleavage site of FMDV, this cleavage sequence differs considerably, in all serotypes sequenced to date, from the consensus sequence of HRV2 and CVB4.

It has been suggested that FMDV L has homology to the papain-like family of cysteine proteases (Gorbalenya et al., 1991). In support of this observation, E-64, an inhibitor of this family of proteases, blocked both *cis* and p220 cleavage by FMDV L (Fig. 3; Kleina and Grubman, 1992). In contrast, the 2A proteases of picornaviruses appear to be homologous to the small bacterial serine proteases (Bazan and Fletterick, 1988; Gorbalenya et al., 1989) and E-64 has no effect on their activity. It will be interesting to identify the active site residues of FMDV L proteinase, since this information and knowledge of its cleavage specificity on the viral polyprotein and p220 should allow rational design of antiviral compounds that are effective and more specific than E-64.

After this manuscript was submitted for publication, Kirchweger et al. (1994)

demonstrated that FMDV Lb cleaves p220 at a different site than HRV2 and CVB4 2A. Furthermore, this site is different from the cleavage site of Lb on the viral polyprotein.

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